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TITLE: RNASEH2A -- a Putative “Non-Oncogene Addiction” Gene Target and Marker for Radio-sensitivity in High Risk Prostate Cancer

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**14. ABSTRACT**
.We proposed that RNASEH2A represents a novel type of gene, up-regulated in lethal prostate cancer to prevent catastrophic genomic instability and cell death and thereby acting to make prostate cancers resistant to treatment with radiation therapy. The major findings to date include (1) independent validation of the associate of RNASEH2A with tumor grade. (2) Identification and sample preparation of RNA specimens with lethal potential for analysis of RNAseH2a expression.(3)Observation that RNASH2A expression does not independently predict lethal prostate cancer.(4)Observation that RNASH2A expression does predict radio-sensitivity and response to treatment in men who underwent radical prostatectomy and subsequently had post – operative radiation. (5) Analysis of prostate cells lines for endogenous expression of RNASH2a (6) construction of plasmids that allow to over express RNAseH2a in prostate cancer cell lines to evaluate their impact on radiation sensitivity.

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High Risk prostate cancer, radio sensitivity, biomarker

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## Introduction

Over the last two decades there have been major advances in both the detection and treatment of localized prostate cancer. Although this has resulted in a decrease in prostate cancer specific mortality, prostate cancer still remains the second leading cause of cancer related death among men. Thus, key issues in the management of prostate cancer today include the identification of men with aggressive disease and the development and improvement of therapies to treat lethal cancers. Currently, Gleason grade is the most potent forecaster of metastatic ability and prostate cancer specific mortality. As such, we have begun to investigate the molecular features of high risk prostate cancer by correlating Gleason grade with RNA based expression patterns. Our work has identified pathways of genome stability as enriched in high grade disease and specifically *RNASEH2A*, a putative chromosomal integrity determinant as one of the most strikingly over-expressed genes in aggressive prostate cancer (over 6 fold increase in Gleason 8 vs Gleason 6 tissue, $p < 1x10^{-6}$). In this proposal, we hypothesized that *RNASEH2A* is associated with lethal, high grade disease maintaining chromosomal stability. Together this proposal implicates *RNASEH2A* as a marker and modulator of radio-resistance in prostate cancer.

## Body

We proposed to in Specific Aim 1 to demonstrate the association of *RNASEH2A* with lethal prostate cancer. To establish this correlation we will examine the expression of *RNASEH2A* RNA and protein in the prostatectomy tissue of men who either rapidly progressed to metastasis and death following local therapy or those with high grade disease and favorable clinical outcomes.

The cohort which we derived cases from are summarized in Table 1. This cohort of men undergoing radical prostatectomy at Johns Hopkins Hospital have know clinical outcomes. To date we have laser capture microdissected index tumors of men with high risk disease and the above mentioned disparate clinical outcome. We have isolated RNA from the formalin fixed, paraffin embedded prostatectomy samples from men found to have Gleason $\geq$8 disease at prostatectomy as we have previously described and outlined in our proposal[1]. We are in the process of performing differential expression of *RNASEH2A* in these men.

In addition, we are validating RNAseH2a expression in a similar cohort to the Johns Hopkins Cohort in collaboration with GenomeDx and the Mayo Clinic. The expression analysis was performed as described. We have published on this dataset within the last year – reference 2. This dataset consists of 235 men with intermediate to high risk prostate cancer who underwent radical prostatectomy at the Mayo Clinic and had long term follow-up. The outcomes of these men are shown in Figure 2. In this cohort, RNAseH2a expression correlates with Gleason score (AUC 0.66, P,0.05) (Figure 1) which is consistent with the original association of RNAseH2a we noted with high grade disease (6 fold over expression in Gleason 8 vs Gleason 6 disease ($p <0.008$). RNAseH2a expression was not associated with biochemical (Figure 2) or metastatic progression (Figure 3) or death (Figure 4) in univariate analysis ($p = 0.7$, 0.15, and 0.6 repectively). The association of RNASEH2A with clinical outcomes in prostate (or any other cancer type) has not yet been reported.

In Aim1 we also proposed to explore the association of RNASEH2A protein and with cancer recurrence. We have developed staining conditions that permit RNASEH2A protein staining radical prostatectomy specimens (Figure 5) and have successfully used them in test TMAs (tissue microarrays). Year 2 aims also include assaying RNASEH2A expression in the Johns Hopkins Progression TMA.

We have begun to examine the effects of *RNASEH2A* overexpression and suppression on cell function, DNA integrity and radiosensitivity in prostate cancer. We initiated these aims by documenting RNASEH2A expression in existing prostate cancer cell lines. RNASEH2A is overexpressed in all prostate cancer cell lines we have tested (Figure 6). This includes both androgen sensitive LnCap and insensitive lines (22RV1, Du145, LaPc4 and PC3). The benign prostate cell line PreC expressed low levels. To explore the effects of RNASEH2A levels on cell function and integrity we have tested constructs to suppress and over express it. To date we have tested 12 different siRNA constructs and have not been able to suppress RNASEH2A protein levels. We are moving into shRNA constructs that have also been published[3] (ON-TARGETplus SMARTpoolDharmacon ) in an effort to suppress it. We have successfully generated constructs to overexpress RNASEH2A with a CMV promoter (Figure 7). Immunohistochemical staining of cell blocks from over-expressing cell lines confirmed the western analysis (Figure 7). Staining was performed with Sigma Aldrich antibody.

In specific aim 3 we proposed that RNAasH2a is a marker of radio-sensitivity. To preliminarily investigate this proposal, we evaluated RNAseH2a expression in 96 men in the Mayo Clinic cohort who underwent adjuvant or salvage radiation. The clinical characteristics of this cohort are described in Table3. We evaluated the association of RNASHH2A expression and biochemical or clinical (metastatic) progression or death in both univariate and multivariate analysis. In this subset, men with high expression of RNASEH2A were statistically more likely to experience biochemical recurrence (Figure 8, $p=0.005$), metastasis (Figure 9, $p = 0.002$) and death (Figure 10, $p = 0.02$) On multivariate analysis incorporating PSA, Gleason score, Seminal vesicle invasion, Extraprostatic extension and surgical margin status, RNASH2A expression significantly predicted biochemical and metastatic free survival ($p <0.001$ for both). Thus

although RNASH2A expression does not appear to predict cancer recurrence outcomes after radical prostatectomy, in the subset of men who underwent post-operative radiation levels of higher levels of expression of RNASH2A does appear to be associated with poorer outcomes in a statistically significant manner.

## Key Research Accomplishments

- RNASH2A expression correlates with prostate cancer grade
- RNASH2A expression does not independently predict lethal prostate cancer in an group of men who underwent radical prostatectomy
- RNASH2A expression does appear to predict radio-sensitivity and response to treatment in men who underwent radical prostatectomy and subsequently had post – operative radiation.
- RNASH2A expression is increased in prostate cancer cell lines and appears to be independent of androgen dependency.

## Reportable Outcomes

**No reportable outcomes have come from this work thus far**

## Conclusions

To date we have demonstrated *RNASEH2A* is associated with high grade prostate cancer but is not an independent marker of lethal disease in men undergoing radical prostatectomy. However in men who underwent radical prostatectomy and experienced a recurrence, RNASH2A expression is a independent marker of radio-resistance in prostate cancer and worse clinical outcome.

## References

1. Ross AE, Marchionni L, Vuica-Ross M, Cheadle C, Fan J, Berman DM, Schaeffer EM., Gene expression pathways of high grade localized prostate cancer., Prostate. 2011 Feb 25.

2. Hurley PJ, Marchionni L, Simons BW, **Ross** AE, Peskoe SB, Miller RM, Erho N, Vergara IA, Ghadessi M, Huang Z, Gurel B, Park BH, Davicioni E, Jenkins RB, Platz EA, Berman DM, **Schaeffer EM**. Secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) is down regulated in aggressive prostate cancers and is prognostic for poor clinical outcome. Proc Natl Acad Sci U S A. 2012 Sep 11;109(37):14977-82. Epub 2012 Aug 27.

3. Crow YJ, Leitch A, Hayward BE, et al: Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection. Nat Genet 38:910-6, 2006

## Appendicies

| Gleason Grade 8-10: | No Recurrence at 5 years (N=92) | Metastasis at 5 year (N=73) |
|---|---|---|
| **Age (yr)** | 59.5 (46-73) | 59 (45-71) |
| % Caucasian | 91% | 86% |
| **RP Gleason** | | |
| 8 | 64 (70%) | 28 (38%) |
| 9 | 28 (30%) | 45 (62%) |
| **Pathologic Stage** | | |
| T2N0Mx | 48 (52%) | 10 (14%) |
| T2N1Mx | 0 (0%) | 0 (0%) |
| T3aN0Mx | 34 (38%) | 11 (15%) |
| T3aN1Mx | 1 (1%) | 7 (9%) |
| T3bN0Mx | 9 (10%) | 24 (33%) |
| T3bN1Mx | 1 (1%) | 21 (29%) |
| Positive SM | 12 (13%) | 25 (34%) |
| **Subsequent BCR** | 25 (27%) | NA |
| **Died From Prostate Cancer** | 0 (0%) | 42 (58%) |
| **Year of Metastasis** | No Metastasis | 3 (1-5) |
| **Year of BCR** | 7 (5-17) | 1 (1-4) |
| **Length Follow-Up** | 8 (5-17) | 6 (1-14) |

Table 1 **Pathological and clinical characteristics of subgroups of men with Gleason 8-10 disease at prostatectomy.** "No

Recurrence" includes men with no PSA recurrence, metastasis or death at 5 years. NA = not applicable

| | BCR | MET | PCSM | GS > 7 | SMS | Adj RTx | Late RTx |
|---|---|---|---|---|---|---|---|
| % | 53 | 31 | 14 | 41 | 57 | 10 | 31 |

Table _. The percentage of biochemical recurrence (BCR), metastatic progression (MET), prostate cancer specific mortality (PCSM), Gleason score (GS), positive surgical margins (SMS), adjuvant radiation therapy (Adj RTx), and salvage radiation therapy (Late RTx) events in the dataset (n = 235).

- Metastatic progression is defined by a positive bone or CT scan.

Table 2 **Clinical follow up of Mayo Clinic Validation set.**

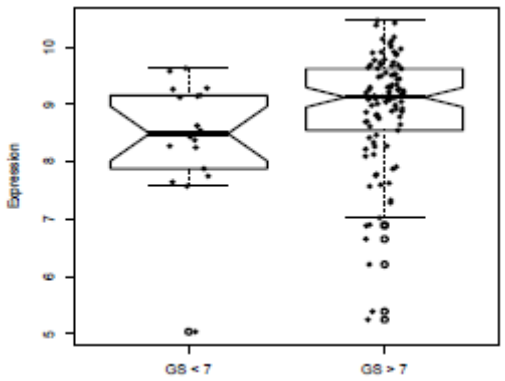


Figure 1. Boxplot of RNAseH2a expression by Gleason score (Gleason 6 vs Gleason 8). ($p < 0.03$)

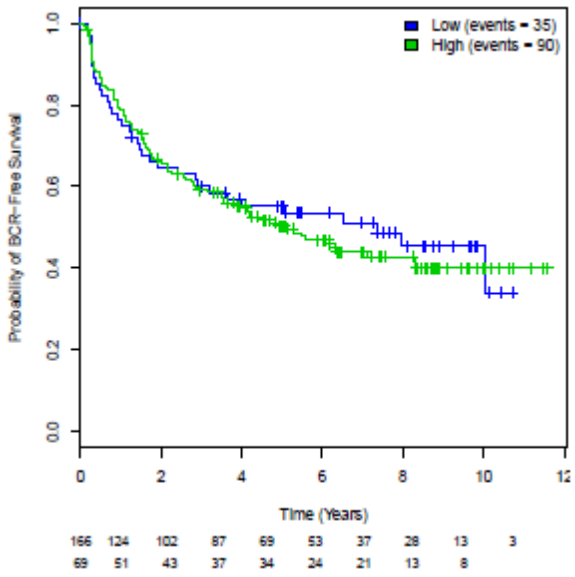

Figure 2 Kaplan Meier curve showing differences in biochemical free survival in patients with high and low RNASH2a expression. (p = 0.722)

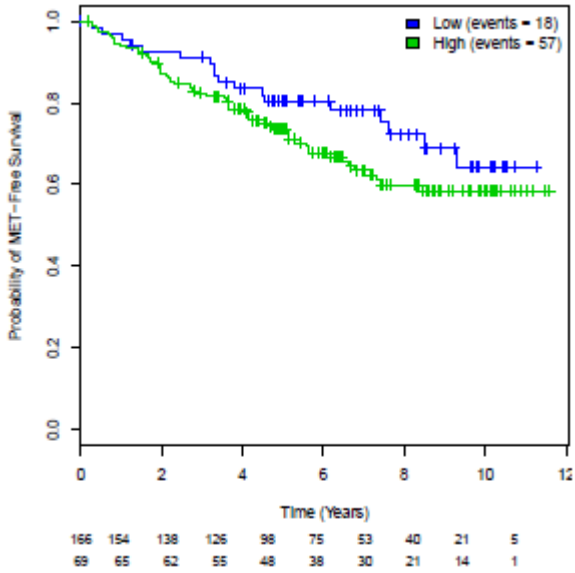


Figure 3. Kaplan Meier curve showing differences in metastatic free survival in patients with high and low RNASH2a expression. (p = 0.158)

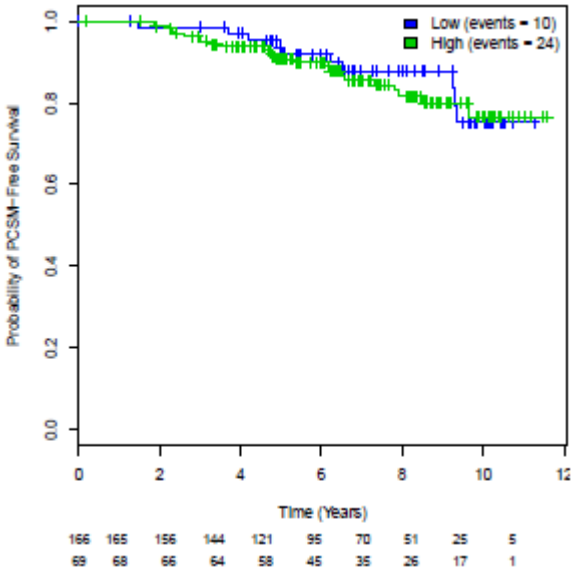


Figure 4. Kaplan Meier curve showing differences in Prostate cancer specific mortality free survival in patients with high and low RNASH2a expression. (p = 0.677)

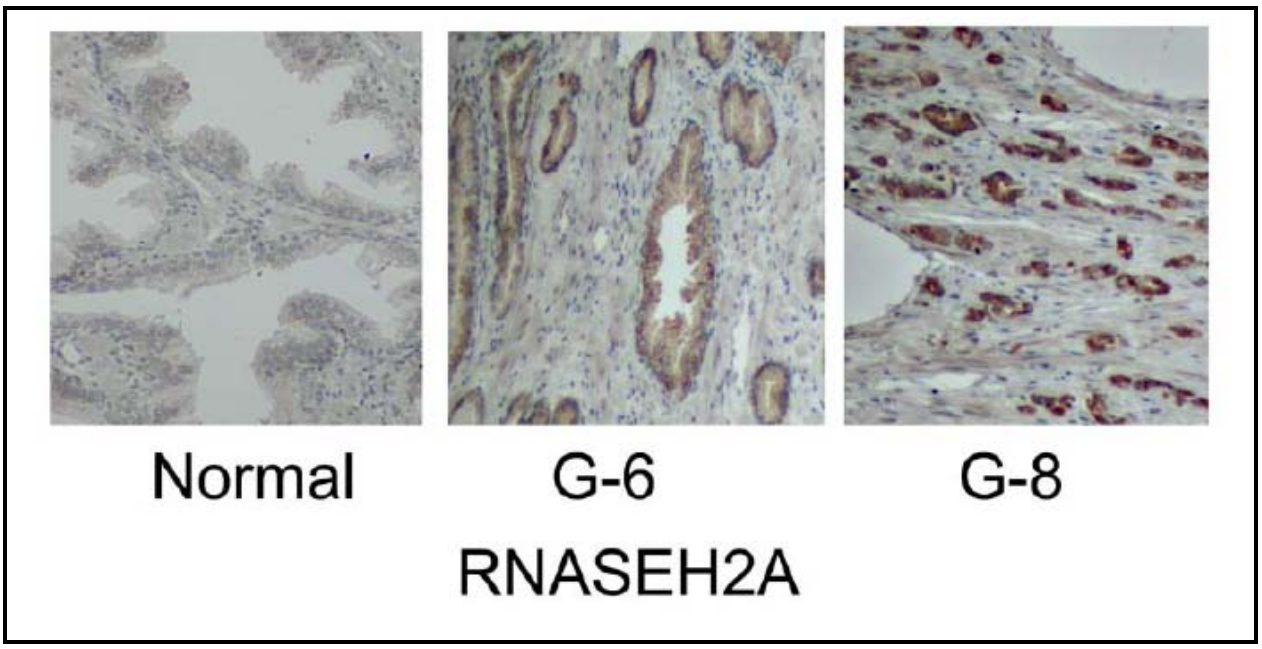


Figure 5. RNASH2A protein is over expressed in human prostate cancers. Immunohistochemical analysis of human prostate cancers show increasing staining with increasing grade of the cancer. G-6 = Gleason 6, G-8 = Gleason 8.

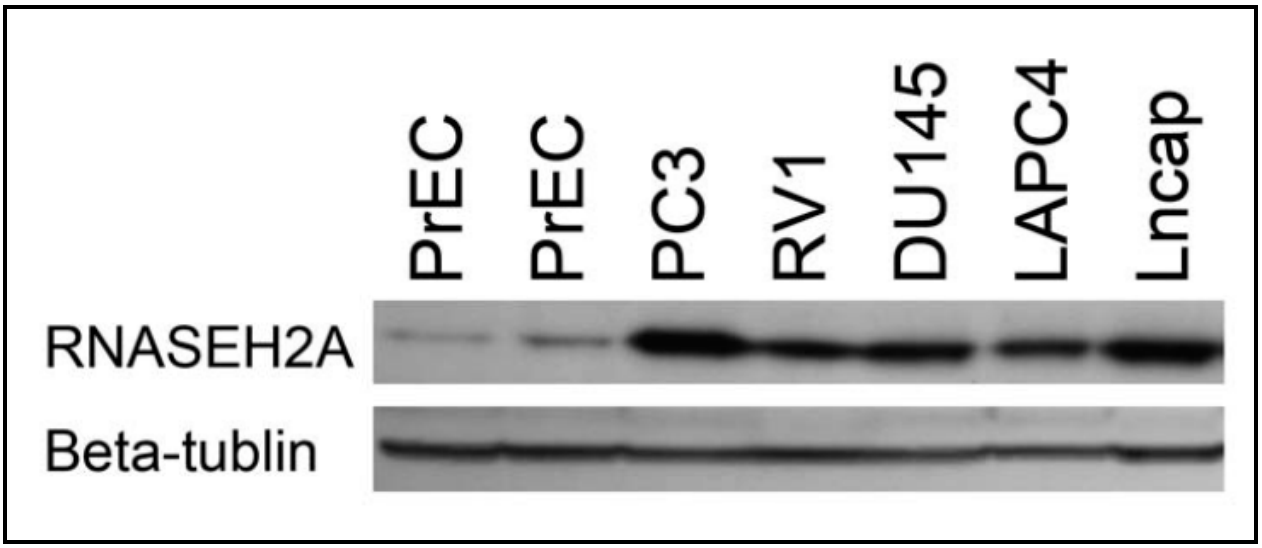


Figure 6. Expression of RNASH2A in benign and malignant prostate cancer cell lines.

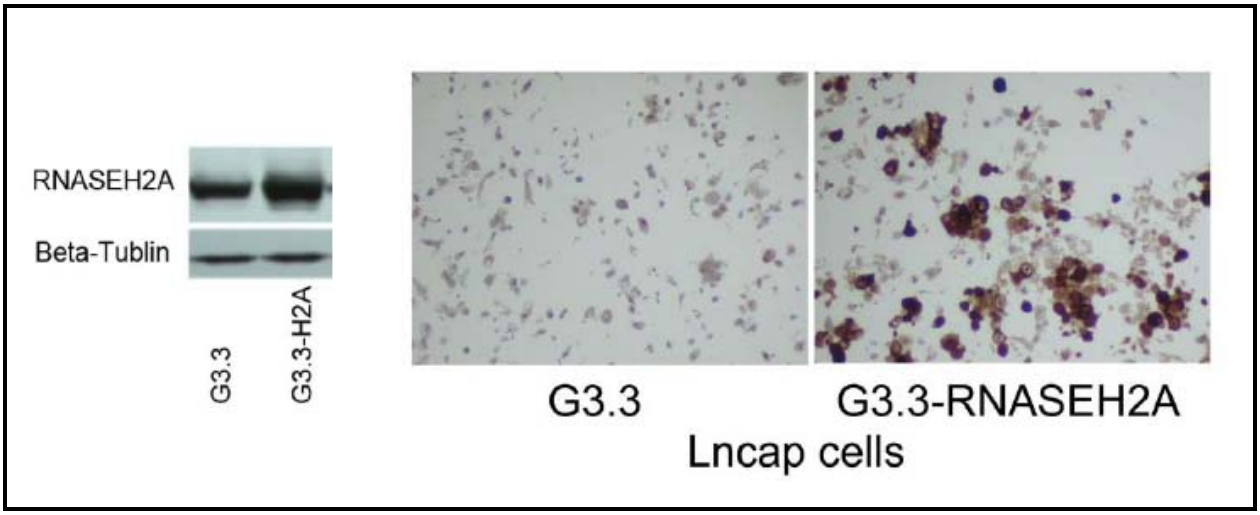

Figure 7. CMV driven overexpresion constructs (PCDNA3.1 backbone) increased RNASH2A protein production. Left panel demonstrated overexpresion by western analysis. Right panel demonstrates over expression by immunohistochemical analysis of 22RV1 cell blocks. G3.3 – parental vector- PCDNA3.1, G3.3-RNASH2A0 overexpression construct.

| | BCR | MET | PCSM | GS > 7 | SMS | Adj RTx | Late RTx |
|---|---|---|---|---|---|---|---|
| % | 76 | 48 | 21 | 38 | 71 | 25 | 77 |

The percentage of biochemical recurrence (BCR), metastatic progression (MET), prostate cancer specific mortality (PCSM), Gleason score (GS), positive surgical margins (SMS), adjuvant radiation therapy (Adj RTx), and salvage radiation therapy (Late RTx) events in the dataset (n = 96).

- Metastatic progression is defined by a positive bone or CT scan.

Table 3. Clinical characteristics of men undergoing radiation in the Mayo Clinic cohort.

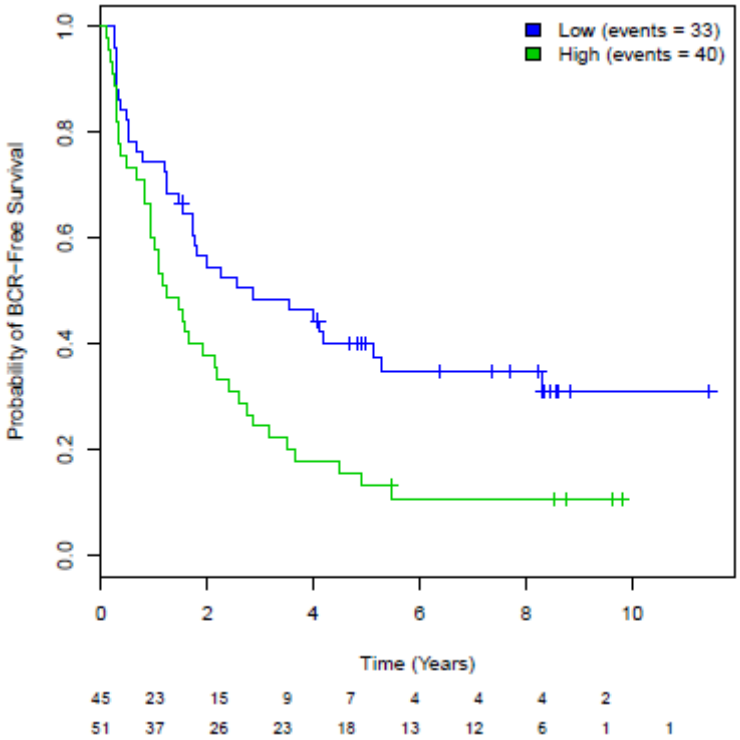


Figure 8. Kaplan Meier curve showing differences in biochemical free survival in patients who had a radical prostatectomy and subsequently underwent post-operative radiation based on high and low RNASH2a expression. (p = 0.005)

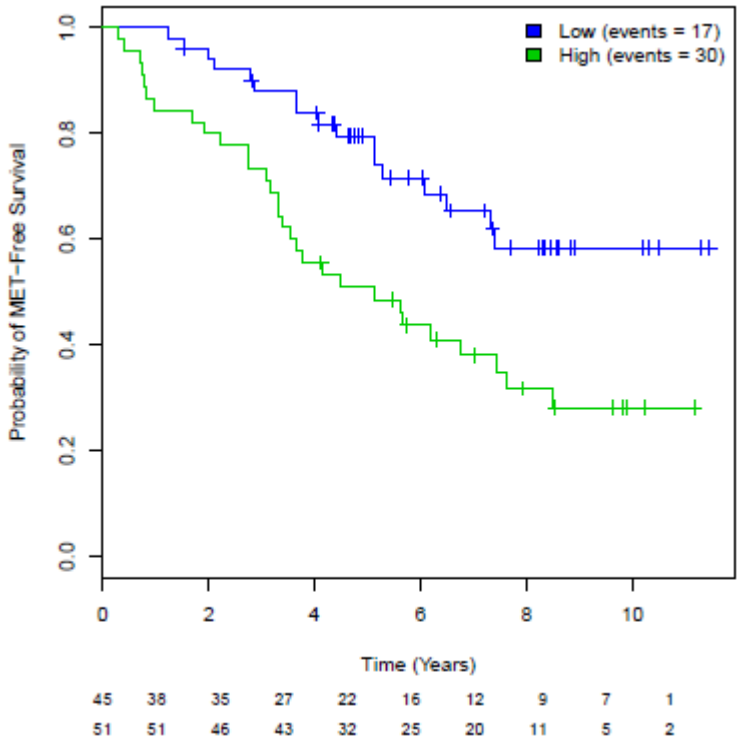


Figure 9. Kaplan Meier curve showing differences in metastasis free survival in patients who had a radical prostatectomy and subsequently underwent post-operative radiation based on high and low RNASH2a expression. (p = 0.002)

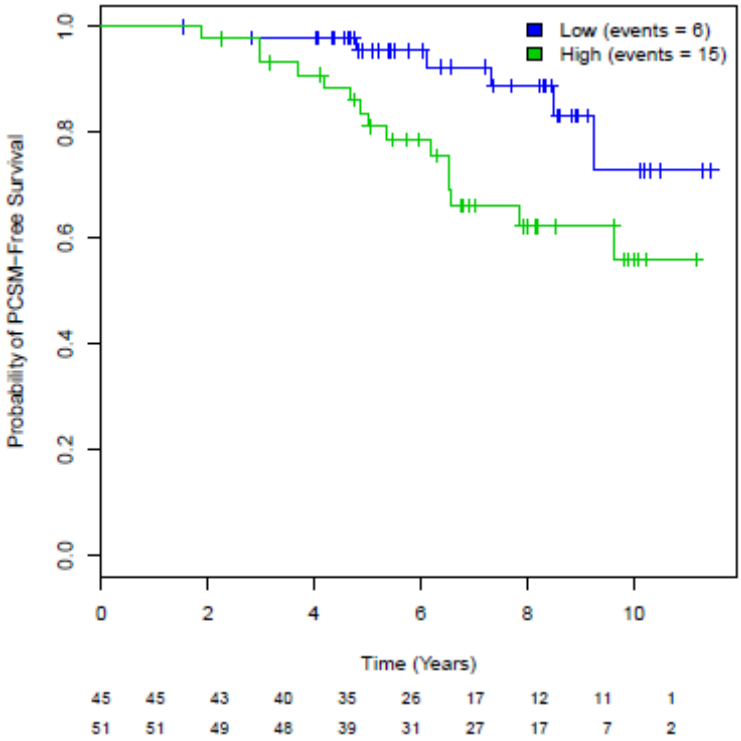


Figure 10. Kaplan Meier curve showing differences in prostate cancer specific survival in patients who had a radical prostatectomy and subsequently underwent post-operative radiation based on high and low RNASH2a expression. (p = 0.024)